CODING FORM FOR SRC INDEXING REVISED 10/15/86

Microfiche No.
OTS0515267

New Doc I.D.
86-870000829

Old Doc I.D.

Date Produced
8/01/82

Date Recieved
6/16/86

TSCA section
8D

Submitting Organization
INTL ISOCYANATE INST

Contractor
MITSUBISHI-KASEI INST

Document Title
MICRONUCLEUS TEST ON 4,4'-MDI - OFFICIAL ENGLISH TRANSLATION
OF JETOC REPORT WITH COVER LETTER DATED 061086

Chemical Category
MDI (101-68-8)

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10th June, 1986

To: Safety Committee
E-AB Sub committee
NA-AB Sub committee

Dear Colleague,

Micronucleus Test on 4,4'-MDI
Official English Translation of JETOC Report

I enclose an official English translation (May 1986) of work carried out in Japan for JETOC by Mitsubishi-Kasei Institute of Toxicological and Environmental Science. In the experiments of this study MDI dissolved in DMSO, then suspended in corn oil. The report concludes, inter alia, that 4,4'-MDI did not cause the production of micronuclei under the experimental conditions, and that it is considered not to induce in vivo chromosomal aberration.

The above outline conclusion had already been communicated to the EPA (see CHIP for MDI, 28 June 1984 p 14) as unpublished work. I have asked that the report be submitted to the EPA for their records.

Yours sincerely,

David S.Gilbert

Enc.

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Micronucleus Test

(hexamethylenetetramine
4,4'-diphenylmethane-diisocyanate)

August, 1982

Japan Chemical Industry Ecology - Toxicology &
Information Center - JETOC

JETOC,
13-4, 1-chome Toranomon,
Minatoku, Tokyo 105,
Japan

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Micronucleus Test

1. Introduction

This micronucleus test was developed as an in vivo test to examine the chromosomal aberration-inducing function of a chemical substance by Heddle and Schmid et al.[2) 3)] The method has an advantage : the examiner can observe micronuclei, which are produced by chromosomal aberration-inducing function and inhibition of spindle-function, relatively easily and infer those functions, instead of directly examining chromosomal aberration, which requires experience.

For the test, mice were used. From mice treated with the test substance, bone marrow cells were obtained and the smear was prepared to examine for the incidence of erythrocytes with micronuclei. Bone marrow cells were collected 24 hours after treatment.

As the positive control, triethylenemelamine (TEM), which had already been confirmed to have mutagenicity (reverse mutation and chromosomal aberration) and carcinogenicity, was used.[4) 5) 6)]

Dosage was selected in reference to the results of preliminary acute toxicity test LD_{50}*.

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* LD_{50} means the dose level which kills half of the animals within 7 days.

2. Materials and Methods

a) Materials

a-1) Experimental animals: 8-week-old male ICR mice were purchased from the Shizuoka Agricultural Cooperative Association for Experimental Animals, and after an acclimatization period of more than one week, those ten weeks old (weighing approximately 40 g) were used for the experiment.

a-2) Preparation of test materials: As it is easily soluble in water (1 g/1.5 ml), hexamethylenetetramine was dissolved in distilled water and sterilized by filtering (0.45μm) for use.

4,4'-diphenylmethane-diisocyanate (MDI) is insoluble in water and reacts with it to form polymers. Therefore, the test material was dissolved in a small amount of dimethylsulfoxide (DMSO, Junsei Chemicals Co., Ltd.), the first solvent, which does not contain water, and then it was suspended in the sterilized corn oil (Kishida Chemicals Co., Ltd.), the second solvent. The preparation was made separately for each test and used immediately.

a-3) Control: For the negative control, the solvents used in the test were employed.

For positive control, TEM (Poly Science Co., Ltd.) was dissolved in distilled water and sterilized by filtering.

a-4) Maintenance: The animals were divided into groups of 5 to 6 mice and housed in large alumilite, lunch box-shaped cages, which were maintained at 2 ± 1°C, room temperature, and 55 to 60%, relative humidity. All maintenance apparatus were sterilized before use, and participants in the experiment used a mask, sterilized working clothes and rubber gloves to prevent infection of the animals.

Commercial mouse pellets (Oriental Yeast Co., Ltd.) and tap water after ultraviolet sterilization were given ad libitum.

The animals were marked on the tail for identification before each experiment, and after weighing, they were assigned to each group in such a way as not to show significant variation in weight between groups.

b) Test Methods

b-1) Dosage

To decide the dose level for the micronucleus test, a one-week acute toxicity test was carried out as

a preliminary test.

Groups of 5 to 6 mice were treated with the test material at different dose levels, and LD_{50} was estimated from the number of deaths.

Dose levels chosen for the micronucleus test were 1/2 of LD_{50} as the maximum and three lower levels. However, if there is some reason, e.g., the test material should not have been the usual toxicity, dose levels were a altered suitably.

b-2) Administration

Mice were administered i.p. with the test material, 10 ml/kg of body weight, by a single injection.

b-3) Preparation of bone marrow smear

Bone marrow cell smear was prepared as follows: Twenty four hours after administration of the test material, mice were killed by cervical dislocation; the femur was excised; after both ends were cut, bone marrow cells were washed out with 0.2 ml of bovine-fetus serum. The collected cell suspension was centrifuged at 1,000 r.p.m. for 5 min; the supernatant was discarded and the residual bone marrow cells were suspended again in a small amount of serum. A drop of the cell suspension was placed and spread on a slide glass, and the smear thus

prepared was dried with blown air or dried overnight.

b-4) Staining

The bone marrow smear preparation was fixed in absolute (99%) methanol for 5 min., stained with 50% May-Grünwald solution (Merck Co., Ltd.) diluted with Sörensen-buffer at pH 6.8 for 5 min., and again stained with 2.5% Giemsa stain (Merck Co., Ltd.) diluted with the same buffer for 30 min. The stained smear plate was rinsed in the same buffer, 0.017% acetic acid solution and distilled water; after drying, it was sealed and served as a specimen for observation.

b-5) Observation

Smear specimens were observed in accordance with the method of Schmid et al[7), 8)].

Erythrocytes, 2,000 per mouse, were observed for the incidences of polychromatic erythrocytes (PCE) and of normochromatic erythrocytes (NCE), with micronuclei, and the incidence of PCE in the erythrocytes observed.

The specimen plates were numbered and a blind test was employed. All the specimens were observed by 1,000-fold magnification in oil immersion using a x 100 magnifying objective lens.

b-6) Evaluation

Results were evaluated by t-test of the incidence of PCE and Kastenbaum and Bowman's statistical analysis for significant differences [8].

c) Place of Examination

Testing facility

Name: Mitsubishi-Kasei Institute of Toxicological and Environmental Sciences

Place: 1000 Kamoshidacho, Midori-ku, Yokohama city

Telephone: (045) 962-1211

Testing period

Dec. 21, 1981 to Feb. 5, 1982

3. Results

a) Hexamethylenetetramine

One gram of hexamethylenetetramine is soluble in 1.5 ml of water, 12.5 ml of alcohol and 10 ml of chloroform. Since solubility in water is the highest, water was used as its solvent[9]. In supersaturation, crystals immediately separate out even after being ground in a mortar. Therefore, 0.5 g/ml solution, which can be prepared, was used for administration as the highest dose level.

Results of acute toxicity test are given in Table 1. No toxicity of hexamethylenetetramine was found, nor were symptoms of acute toxicity observed in mice. For the purpose

of detecting the presence of myelopoiesis-inhibiting function, the incidence of PCE in whole erythrocytes was studied, but little inhibition of myelopoiesis was found.

On the basis of the above results, 5,000 mg/kg was decided as the highest dose level in the micronucleus test, and 3 lower levels with the common ratio of 2 were employed. Three levels of concentration were used because the test materials, which are industrial chemicals, do not need to be evaluated for effective concentration as in drugs.

Results of micronucleus test are listed in Tables 2 and 4.

In the positive control group (treated with TEM), the incidence of PCE decreased to approximately 50% of that in the control, suggesting the existence of inhibitory action against increase of bone marrow cells. The number of erythrocytes with micronuclei conspicuously increased.

In treated groups, especially in the 5,000 mg/kg group, the incidence of PCE decreased to approximately 77% of that in the control, and proliferation of bone marrow cells was slightly but not significantly inhibited as in other groups.

The number of erythrocytes with micronuclei increased in PCE and whole erythrocytes in all the groups; however, no significant difference was detected by Kastenbaum and Bowman's test.

b) 4,4'-diphenylmethane-diisocyanate (MDI)

Since it is insoluble in water and produces polymers by reacting with it, MDI was dissolved in a small amount of dehydrated DMSO as the primary solvent. This was suspended in sterilized corn oil, the second solvent, and immediately used for the test.

DMSO was used in the concentration of 0.04 to 0.05% (final concentration) without toxic effect. Results of the acute toxicity test are shown in Table 1. LD_{50} was estimated to be about 100 mg/kg from tests repeated three times.

As a result, the highest dose level for the micronucleus test is ordinarily 50 mg/kg, 1/2 of LD_{50}; presently, however, the highest dose level was selected on the following basis:

MDI, as mentioned above, reacts with water to produce polymers which are insoluble in water, and it has a corrosive effect on tissues (skin, eyes, digestive tract, etc.) and causes permanent damage to them[10]. Actually, autopsies of dead mice revealed, in almost all the cases, intraperitoneal cementification of MDI and thus induced adhesion of the intestinal tract and solidified state. Mice which died were those treated with 133 to 500 mg/kg of the test material; death occurred in a similar fashion in all the cases and no death occurred within one day. The main cause of death with MDI, therefore, was not considered to be a systemic effect caused by absorption but physical damage. Therefore,

200 mg/kg, which is close to half of the lethal dose level, was decided as the highest dose level, and three lower levels with a common ratio of 2.5 were employed. The reason for taking 3 dose levels is the same as that in the use of hexamethylenetetramine.

Results of micronucleus tests using the above dose levels are shown in Tables 3 and 4.

In negative controls, the group treated with distilled water and that treated with 4% DMSO-contained corn oil, the incidence of PCE in the latter decreased to approximately 75% of that in the former group and the number of erythrocytes with micronuclei increased 3-fold, suggesting the presence of some effect. No significant difference was detected by statistical test.

In the positive control using TEM, the incidence of PCE decreased to 55% of that in the negative control (treated with distilled water) and suggested inhibited myelopoietic function of th bone marrow. Erythrocytes with micronuclei were markedly increased. These results showed excellent reproducibility as in the groups treated with hexamethylenetetramine.

Of the treated groups, in the group treated with 200 mg/kg, the incidence of PCE decreased to 67% of that in the negative control (4% PMSO corn oil) with a significant difference; this suggested inhibited myelopoietic function of the bone

marrow. No significant difference was found in any other group.

With the number of erythrocytes with micronuclei, little difference was found between treated groups and the nagative control.

c) Conclusion

The above results show that neither hexamethylenetetramine nor MDI causes micronuclei under the present conditions. These test materials, therefore, are not considered to induce in vivo chromosomal aberration.

Table 1 Preliminary toxicity test

No. of experiment	Test material	Dose levels (mg/kg)	Number of animals which died / Number of animals treated	Time of death (day) 0-1	1-3	3-5	5-7
1	Hexamethylene-tetramine	5,000	0/6	0	0	0	0
		2,500	0/6	0	0	0	0
		1,250	0/6	0	0	0	0
1	MDI	500	5/5	0	3	2	0
		100	0/5	0	0	0	0
		20	0/5	0	0	0	0
		10	0/5	0	0	0	0
2		450	4/6	0	3	1	0
		300	5/6	0	2	1	2
		200	5/6	0	4	1	0
		133	4/6	0	2	0	2
3		200	5/6	0	2	3	0
		133	4/6	0	1	1	2
		89	0/6	0	0	0	0

Table 2 Results of micronucleus test with hexamethylenetetramine in mice

Test material	Dose levels (mg/kg)	Frequency of administration	Route of administration	Number of animals	Incidence of PCE (%)	Observation Erythrocyte counts	Counts of erythrocytes with micronuclei PCE	NCE	Total
Sterilized distilled water (Solvent control)	0	1	i.p.	6	34.8	2,000	0	0	0
					42.0	2,000	0	0	0
					33.4	2,000	0	1	1
					47.0	2,000	1	0	1
					25.5	2,000	1	1	2
					34.8	2,000	1	1	2
Hexamethylene-tetramine	1,250	1	i.p.	6	20.7	2,000	0	0	0
					42.8	2,000	0	0	0
					38.6	2,000	1	0	1
					30.5	2,000	1	1	2
					38.3	2,000	2	0	2
					39.3	2,000	1	1	2
	2,500	1	i.p.	6	33.9	2,000	1	0	1
					29.6	2,000	0	2	2
					29.9	2,000	2	0	2
					44.1	2,000	2	0	2
					39.0	2,000	3	0	3
					30.8	2,000	3	1	4
	5,000	1	i.p.	6	39.2	2,000	0	0	0
					22.3	2,000	1	0	1
					18.1	2,000	2	0	2
					20.6	2,000	0	3	3
					26.5	2,000	3	0	3
					39.9	2,000	3	0	3
TEM (Positive control)	0.5	1	i.p.	6	16.4	2,000	23	0	23
					17.6	2,000	24	3	27
					16.6	2,000	27	2	29
					16.9	2,000	30	1	31
					22.3	2,000	38	3	41
					27.8	2,000	55	3	58

Table 3 Results of micronucleus test with MDI in mice

Test materials	Dose levels (mg/kg)	Frequency of administration	Route of administration	Number of animals	Incidence of PCE (%)	Observation Erythrocyte counts	Counts of erythrocytes with micronuclei PCE	NCE	Total
Sterilized distilled water (Solvent control)	0	1	i.p.	6	34.6	2,000	0	0	0
					45.2	2,000	0	0	0
					45.9	2,000	0	0	0
					35.4	2,000	1	0	1
					39.9	2,000	1	0	1
					47.2	2,000	1	0	1
Sterilized corn oil (Solvent control)	0	1	i.p.	6	34.6	2,000	0	0	0
					25.4	2,000	1	0	1
					29.9	2,000	0	1	1
					29.9	2,000	0	2	2
					30.6	2,000	1	1	2
					31.4	2,000	2	1	3
MDI	32	1	i.p.	6	29.8	2,000	0	0	0
					22.2	2,000	0	1	1
					26.8	2,000	1	0	1
					30.0	2,000	1	0	1
					31.5	2,000	0	1	1
					21.1	2,000	2	0	2
	80	1	i.p.	6	22.4	2,000	0	0	0
					37.2	2,000	0	0	0
					37.2	2,000	0	0	0
					21.7	2,000	1	0	1
					28.0	2,000	1	1	2
					33.5	2,000	2	1	3
	200	1	i.p.	6	30.1	2,000	0	0	0
					13.9	2,000	1	0	1
					15.6	2,000	1	0	1
					18.4	2,000	0	1	1
					23.7	2,000	1	1	2
					19.8	2,000	1	2	3

(Continued)

(Continued)

Test materials	Dose levels (mg/kg)	Frequency of administration	Route of administration	Number of animals	Incidence of PCE (%)	Observation Erythrocyte counts	Counts of erythrocytes with micronuclei PCE	NCE	Total
TEM (Positive control)	0.5	1	i.p.	6	16.1	2,000	18	2	20
					24.8	2,000	28	0	28
					24.8	2,000	29	0	29
					16.0	2,000	31	1	32
					26.9	2,000	33	5	38
					28.8	2,000	52	2	54

Table 4 Results of micronucleus test in mice

Test materials	Dose levels (mg/kg)	Frequency of administration	Route of administration	Number of animals	Incidence of PCE[b] (mean %±s.e.)	Observation Erythrocyte counts	Counts of erythrocytes with micronuclei[c] (mean ‰) PCE	NCE	Total
Sterilized distilled water (Solvent control)	0	1	i.p.	6	36.25±3.04	12,000	3 (0.25)	3 (0.25)	6 (0.5)
Hexamethylene-tetramine	1,250	1	i.p.	6	35.03±3.31	12,000	5 (0.42)	2 (0.17)	7 (0.58)
	2,500	1	i.p.	6	34.55±2.39	12,000	11 (0.92)	3 (0.25)	14 (1.17)
	5,000	1	i.p.	6	27.76±3.89	12,000	9 (0.75)	3 (0.25)	12 (1)
TEM (Positive control)	0.5	1	i.p.	6	19.6 ±1.87*	12,000	197**(16.42)	12 (1)	209**(17.42)
Sterilized distilled water (Solvent control)	0	1	i.p.	6	41.37±2.26	12,000	3 (0.25)	0 (0)	3 (0.25)
Sterilized corn oil[a] (Solvent control)	0	1	i.p.	6	30.3 ±1.21	12,000	4 (0.33)	5 (0.42)	9 (0.75)
MDI	32	1	i.p.	6	26.9±1.18	12,000	4 (0.33)	2 (0.17)	6 (0.5)
	80	1	i.p.	6	25.6±2.70	12,000	4 (0.33)	2 (0.17)	6 (0.5)
	200	1	i.p.	6	20.25±2.41*	12,000	4 (0.33)	4 (0.17)	8 (0.67)
TEM (Positive control)	0.5	1	i.p.	6	22.9±2.25*	12,000	191**(15.92)	10**(0.83)	201**(16.75)

a. Sterilized corn oil contains 4% DMSO.
b. t-test; * significantly different ($p < 0.01$)
c. Kastenbaum and Bowman's statistical method; ** significantly different ($p < 0.01$)

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